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13. ABSTRACT (Maximum 200 Words)
Our laboratories have developed and utilized cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. We hypothesize that, due to the stringency of telomerase repression in humans, attaining these defects may be rate-limiting in human carcinogenesis. Our goal is to define the minimum number of genetic and epigenetic changes that permit telomerase reactivation and immortal transformation of finite lifespan HMEC, *in a manner that models changes observed in breast cancers in vivo*. Thus far, we have been able to obtain immortalized HMEC using a combination of two oncogenes (c-*myc* and ZNF217) with pathological relevance to human breast cancer. Although CGH analyses of some of these immortal lines did not show any detectable large-scale changes in gene copy numbers, these lines have all undergone clonal selection, suggesting that unknown stochastic changes, in addition to over-expression of c-*myc* and ZNF217, might be necessary for immortalization. We are currently working to identify these stochastic changes. Better understanding of the underlying molecular changes involved in telomerase reactivation may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

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INTRODUCTION

Immortality allows the progeny of a single cell to accumulate the multiple errors needed to gain invasive and metastatic properties. Long-lived animals such as humans have developed extremely stringent mechanisms of cellular replicative senescence to prevent immortal transformation, presumably as a tumor-suppressor mechanism. Many recent studies suggest that one pathway by which stringent senescence is enforced in normal human cells is through repression of hTERT expression and telomerase activity. Normal human breast tissues show no detectable telomerase activity, while almost all breast cancers express hTERT and telomerase activity. Our laboratories have developed and utilized cultured human mammary epithelial cells (HMEC) to gain information on the defects in growth control processes that allow finite lifespan HMEC to overcome all senescence barriers, reactivate telomerase, and gain immortal potential. Our previous work demonstrated that cultured HMEC could be immortally transformed following exposure to combinations of *pathologically relevant* oncogenic agents. All of these studies generated immortal lines containing unknown errors. We proposed in this grant that the telomere dysfunction-based senescence barrier could be overcome by reactivation of hTERT, but that doing so required the cells to undergo changes in multiple distinct pathways. This requirement may be responsible for the stringency of telomerase repression in humans, and consequently, reactivating telomerase may be a key rate-limiting step in human carcinogenesis. Our previous studies suggested specific defined defects that might be involved in telomerase reactivation. The goal of this project has been to determine the specific genetic and epigenetic changes that permit hTERT reactivation and immortal transformation of finite lifespan HMEC, *in a manner that models changes observed in breast cancers in vivo.*

BODY

Task 1. Perform a semi-quantitative assessment of the efficiency of HMEC immortalization and associated changes in phenotype by GSE22, c-*myc*, and *ZNF217* alone or in combination.

Spontaneous transformation to immortality is virtually non-existent in cultured human cells derived from normal tissues. HMEC readily overcome an RB-mediated, stress associated senescence barrier, stasis, through loss of p16 expression. However, continued telomere erosion with ongoing proliferation of these post-stasis cells (termed post-selection where p16 silencing occurred spontaneously, and extended life when p16 loss occurred after carcinogen exposure) eventually produces widespread genomic instability as a consequence of telomere dysfunction (1-4). Our previous data has indicated that multiple alterations are required for p16(-) HMEC to overcome the telomere dysfunction-based senescence barrier (termed agonescence in p53(+) cells; crisis in p53(-) cells). Overcoming this barrier is associated with gaining the capacity to express hTERT. We therefore proposed that at least two alterations are required to reactivate endogenous hTERT in these p16(-) HMEC. The likelihood that all the necessary errors would occur in the same cell, even under conditions where widespread genomic errors are generated, is exceedingly small. However, if the telomere dysfunction barrier were approached in cells already harboring one error predisposing to immortality, the genomic instability resulting from telomere dysfunction could give rise to rare additional complementary errors that allow reactivation of endogenous telomerase activity and immortalization. Defining the errors that allow telomerase reactivation could greatly increase our understanding of the derangements required for human breast cancer progression.

We first introduced individual errors into p16(-) HMEC – over-expression of the breast cancer-associated oncogenes c-*myc* or *ZNF217*, and/or inhibition of p53 function by the genetic suppressor element GSE22. Immortal lines resulting from some of these manipulations all contained some karyotypic errors, and most lines emerged during the period of genomic instability accompanying telomere dysfunction. We then proceeded to use combinations of defined errors to see if we could produce immortal transformation without incurring undefined errors. We hypothesized that such immortalized cells would require changes in a minimum of three distinct pathways, resulting in: (a) the hTERT gene being rendered accessible to transactivation; (b) appropriate transcriptional activator(s) of hTERT being aberrantly expressed; and (c) molecules that inhibit telomerase

activity being functionally eliminated. Post-selection p16(-) HMEC from specimen 184, having no known existing genetic defects, were transduced with retroviral vectors containing c-*myc*, *ZNF217*, and GSE22, alone or in combination. We monitored three lineages of each experimental condition independently to control for jackpot effects and overgrowth by rare variants within a population. Resulting populations were assayed for expression of the transgenes, hTERT mRNA levels, telomerase activity, SA-βgal activity, morphology, growth rates, colony forming efficiency, labeling index ± TGFβ, genome copy number changes (by CGH), mean TRF length, the number of foci of growing cells that appear when most cells have ceased growth at agonescence/crisis, and retroviral integration sites.

In two preliminary experiments, transduction of both *ZNF217* and c-*myc* into post-selection 184 HMEC produced what appeared to be uniform immortalization; CGH analyses of the immortal populations did not show any detectable changes in gene copy numbers (data not shown). In separate, NIH-supported experiments, we also demonstrated that although newly immortal p53(+) HMEC lines were capable of expressing telomerase activity, the functional p53 in these cells was able to repress this activity until telomeres became critically short and a conversion process was initiated (2).

In the past year, we have examined the molecular consequences of transducing post-selection 184 HMEC at passages 7-9 with combinations of *ZNF217*, c-*myc*, and GSE22. Three independent lineages have been monitored for each combination. As reported last year, the combination of over-expressed *ZNF217* and c-*myc* (ZM) produced homogeneously growing immortalized cells in one lineage (ZM3), heterogeneously growing immortalized cells in a second lineage (ZM2), and no immortalized cultures in the remaining lineage (ZM1). *ZNF217* alone (ZB) produced sporadic immortality in 1/3 lineages. No immortal clones arose in any of the three c-*myc* alone (LM) lineages. Assays of telomerase activity showed early reactivation in ZM3, and reactivation during agonescence in ZM2 (**Fig. 1**). CGH analysis showed no changes in gene copy number in ZM3, but amplification of the 8q locus where c-myc resides, as well as other alterations, in ZM2 (**Fig. 2A,B**). 8q was also amplified in the newly generated ZB line, as it was in previously examined lines immortalized with ZNF217 alone.

Additional information consistent with a role of ZNF217 and c-*myc* in HMEC immortalization was generated by related experiments designed to assess the effect of p16/RB repression of cell cycle progression in pre-stasis HMEC still capable of p16 expression. Pre-stasis 184 HMEC were transduced with siRNA to p16 at passage 4. As expected, this population never exhibited stasis. As most of the population showed diminished growth around passage 14, small areas of morphologically distinct cells appeared and eventually demonstrated good growth. CGH analysis of this immortal line indicated its clonal origin. Remarkably, the only two locations of gene copy number increase in this line were at 8q24 - the location of the c-*myc* gene, and 20q13 – the location of the ZNF217 gene (**Fig. 2C**). Further studies will assess c-*myc* and ZNF217 gene expression in this line.

Despite our ability to generate immortal lines without large gene copy number alterations in cultures transduced with ZNF217 and c-*myc,* southern analysis of retroviral integration sites in our three ZM lines with no detectable CGH alterations indicated a limited number of viral integrations, suggesting that clonal selection had taken place in all three lines. We hypothesize that the variable results obtained in the ZM experiments could be due to: (a) variability in the levels of *ZNF217* and c-*myc* expression achieved; (b) epigenetic influences; (c) stochastic genetic events too small to be detected by CGH (e.g. point mutations, small deletions, etc.). To address possibility (a), we are comparing *ZNF217* and c-*myc* transcript and protein levels in the transduced cells prior to and after immortalization. We are currently addressing possibilities (b) and (c) by (i) investigating a possible role of methylation of the hTERT gene, and (ii) gene expression profiling of finite and immortal HMEC transduced with ZNF217 and c-*myc* (described in Technical Objective 2).

Our studies of hTERT gene methylation have been conducted in collaboration with Jean Benhattar (Institut Universitaire de Pathologie, Lausanne, Switzerland), and in coordination with another DOD supported study (DAMDW81XWH-04-1-0283) of a novel DNA binding protein (BORIS) with possible hTERT gene regulatory

activity. Previous studies by Dr. Benhattar have shown a strong positive correlation between methylation of the hTERT gene promoter and telomerase activity in tumors and tumor-derived lines (5). In contrast, however, studies with normal human cells have suggested a negative correlation between hTERT expression and methylation of the hTERT promoter region (6). We have provided Dr. Benhattar with DNA from finite lifespan and immortal HMEC derived from different individuals. His laboratory has examined hTERT promoter methylation by two different methods – methylation-sensitive single-strand conformation analysis (MS-SSCA) and/or a methylation-sensitive DNA dot blot analysis (**Table 1**). Similar to what has been shown for tumor lines, most of the immortal HMEC lines showed methylation of the hTERT promoter. However, we note that most HMEC that immortalized readily, without undergoing the period of genomic instability that accompanies telomere dysfunction (e.g., lines transduced with both ZNF217 and c-*myc*), did not show methylation. This preliminary data therefore does not support the hypothesis that alterations in hTERT gene promoter methylation patterns are an epigenetic factor contributing to immortalization of lines that show no gross genomic changes by CGH. However, the possibility remains that methylation at alternative sites that have not yet been examined in the hTERT promoter or gene body may play such a role. It is also possible that alterations in hTERT gene promoter methylation may only play a role in immortalization of lines that exhibit genetic instability accompanying telomere dysfunction.

Since our previously published work (2) has shown that loss of p53 function can accelerate the acquisition of telomerase activity in susceptible HMEC lines, we performed experiments to determine whether inactivation of p53 can increase the level of telomerase activity and frequency of immortalization in HMEC that initially failed to immortalize when transduced with both ZNF217 and c-*myc*. Specifically, we transduced GSE22 into the ZM1 lineage as well as into ZB and LM lineages (lineages that all did not immortalize). Although the cells transduced with GSE22 did exhibit more population doublings than control cultures prior to agonescence/crisis, we did not observe significant differences in immortalization or telomerase activity, negating the likelihood that p53 contributed to the poor frequency of immortalization.

The absence of gross genomic changes in three of our HMEC lines immortalized by ectopic expression of ZNF217 and c-*myc* suggests that it may be possible to generate malignant HMEC from normal HMEC using known, *pathologically relevant* agents, without the need for unknown errors generated by genomic instability or viral oncogenes. We are thus initiating new studies to determine if we can malignantly transform the ZNF217/c-*myc* immortalized HMEC. These studies may help address the question of whether aneuploidy per se is necessary for malignancy, or whether defined genomic imbalances introduced experimentally are sufficient. Our plan is to over-express additional tumor associated genes into ZNF217/c-*myc* immortalized HMEC. Resulting cultures will be assayed for malignancy-associated properties such as anchorage independent growth and invasiveness. Initial studies will use the wild type and mutant erbB2/Her2/neu oncogene that is amplified/over-expressed in approximately 20-30% of human breast cancers.

Technical Objective 2: Perform differential screening of high density cDNA microarrays to identify genes whose expression is altered in closely related finite lifespan, EL, and immortal HMEC:

To identify gene expression changes that are associated with ectopic expression of ZNF217 and c-*myc*, we have performed expression array analysis comparing finite lifespan 184 HMEC, the 184-ZM3 HMEC prior to the detection of telomerase activity, and fully immortal telomerase(+) 184-ZM3 HMEC. RNA was isolated from duplicate cultures of the indicated cells and analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array in collaboration with Dr. Jeff Gregg at the University of California Davis Medical School. Comparisons of the pre-immortal 184-ZM3 with finite lifespan 184 revealed fewer than 200 transcripts whereas comparisons of the fully immortal 184-ZM3 with the finite lifespan 184 revealed approximately 1300 transcripts showing greater than ±1.6 fold (signal log ratio of ±0.7) differences in expression levels (data not shown). Thus we found that there were further changes in gene expression after immortalization. Changes in gene expression patterns were seen in pathways for integrin signaling, apoptosis, cell cycle regulation, MAP kinase, as well as general metabolic processes. While presumably, most of these additional changes did not directly affect hTERT

expression, analysis of the changes may yield insights into pathways responsible for both the changes observed and hTERT expression.

To help visualize changes in gene expression among the cell types, data has been subjected to cluster analysis (Cluster 3.0). This allows us to identify genes that show similar patterns of expression changes. We have also performed cluster analyses on more limited data sets such as cyclins/cdks/cdkis (**Fig. 3**) and serine/threonine kinases (**Fig. 4**) to determine if transcripts in easily identifiable pathways show coordinate changes. So far this approach has not revealed any strong correlations. We are presently performing additional analyses using the Arrayassist and Pathwayassist programs (Iobion) to identify regulatory pathways that are altered in the presence of the transduced genes and/or after immortalization.

Technical Objective 3: Use random homozygous knockout (RHKO) selection method to identify genes that suppress HMEC immortalization.

No additional experiments related to this objective were conducted in Year 2. The summary of results for Year 1 is repeated below.

In collaboration with Dr. Stanley N. Cohen, we have employed his random homozygous knockout (RHKO) selection strategy to try to identify unknown genes whose inactivation promotes immortalization of post-selection p16(-) HMEC. In three independent experiments, a total of 32 plates were infected with both a tTA vector encoding a doxycycline-sensitive transactivator and an RHKO vector encoding a tTA responsive antisense promoter. In the first experiment, 1/9 plates containing benzo[a]pyrene-treated extended-life184Aa cells yielded a clonal outgrowth with additional proliferative potential. Unfortunately, this outgrowth ultimately senesced before sufficient material could be obtained for further analysis. In a subsequent experiment, post-selection 184 cells were infected with GSE22 to inactivate p53 function prior to infection with the RHKO vector. Despite this modification, all the RHKO-infected cells on 11 plates ultimately underwent crisis, yielding no clones with additional proliferative potential. In the third experiment, we attempted to take advantage of the differential sensitivity of finite life span and immortalized HMEC to *raf* oncogene-induced growth arrest (7) to identify immortalized cells in RHKO-infected cultures of finite life span specimen 48RS. We found unexpectedly that the *raf*-induced growth arrest was not stringent – all 12 plates infected with RHKO and tamoxifen inducible *raf-ER* constructs yielded slowly growing *raf*-resistant cells which ultimately senesced. We are currently re-evaluating the RHKO methodology before attempting any additional experiments.

KEY RESEARCH ACCOMPLISHMENTS

• Immortalized HMEC were obtained using a combination of two oncogenes (c-*myc* and *ZNF217*) with pathological relevance to human breast cancer.

• CGH analyses of three immortal populations obtained using c-*myc* and *ZNF217* did not show any detectable changes in gene copy numbers.

• Southern analysis of viral integration sites has indicated that these three lines have undergone clonal selection, suggesting that unknown stochastic changes, in addition to over-expression of these 2 genes, might be necessary for immortalization.

• Methylation of the hTERT promoter correlates with hTERT expression in immortally transformed HMEC lines that have undergone genetic instability accompanying telomere dysfunction. However, in lines that have not undergone genetic instability, methylation of the hTERT promoter is not required for hTERT mRNA expression.

REPORTABLE OUTCOMES

Telomerase Repression and Telomere Dysfunction in Human Mammary Epithelial Cell Transformation, Paul Yaswen, James Garbe, Betty Gardie, Tarlochan Nijjar, and Martha Stampfer, presented at the International Association for Breast Cancer Research, Sacramento, CA, November 1-5, 2003.

Telomerase Reactivation and Genomic Instability during Immortal Transformation of Cultured Human Mammary Epithelial Cells
Martha Stampfer, James Garbe, Koei Chin, Collin Collins, Joe Gray, Fred Waldman, Karen Swisshelm. Thea Tlsty and Paul Yaswen, presented at the conference on Aneuploidy in Cancer, Society for Investigative Research, Oakland, CA, January 23-26, 2004.

Changes in hTERT gene methylation during immortalization of human mammary epithelial cells
Joanna Mroczkowska, Stéphanie Renaud, Martha Stampfer, Jean Benhattar, and Paul Yaswen, presented at the Keystone Symposim on Epigenetic Regulation, Tahoe City, CA, January 21-26, 2004,

Cooperative Changes Resulting in De-repression of Telomerase and Immortalization of Human Mammary Epithelial Cells
Paul Yaswen, James Garbe, Betty Gardie, Tarlochan Nijjar, Genevieve Nonet, Colin Collins, and Martha Stampfer, presented at the 95th AACR Annual Meeting, Orlando, FL, March 28-31, 2004.

Chin, K, Ortiz de Solorzano, C, Knowles, D, Jones, A, Chou,W, Rodriguez, E, Kuo, W-L, Ljung, B-M, Chew, K, Krig, S, Garbe, J, Stampfer, M, Yaswen, P, Gray, JW, Lockett, SJ. *In Situ* Analysis of Genome Instability in Breast Cancer. Nature Genetics, in press.

Stampfer, M, Garbe, J, Chin, K, Collins, C, Gray, J, Waldman, F, Swisshelm. K, Tlsty T, Yaswen, P. Telomerase Reactivation and Genomic Instability during Immortal Transformation of Cultured Human Mammary Epithelial Cells. Cellular Oncology, in press.

CONCLUSIONS

A high level of telomerase activity is one of the most common distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite life span cells. In the absence of high telomerase levels, cells growth arrest or die due to telomere dysfunction before all the errors necessary for invasive cancer can accrue. Our studies are designed to address the crucial question of what errors are responsible for allowing the telomerase reactivation that transforms finite lifespan cultured HMEC to immortality, *in a manner that models changes observed in breast cancers in vivo*. We believe that understanding how telomerase is reactivated in human cells is of critical significance because; (a) overcoming senescence and attaining immortality may be rate-limiting in human carcinogenesis; (b) human and rodent cells have significant differences in telomere biology - the lack of strict telomerase repression and stringent senescence in rodent cells means that they can not model the human mechanisms. A better understanding of the underlying molecular changes involved in telomerase reactivation may provide novel prevention strategies and/or targets for therapeutic intervention in breast cancer pathogenesis.

The data generated by this grant thus far supports our hypothesis that telomerase is repressed by multiple mechanisms in HMEC. We can repeatedly generate immortal lines lacking detectable genomic copy number alterations by CGH from finite lifespan HMEC transduced with both ZNF217 and c-myc. However, the clonal nature of these lines suggests that over-expression of ZNF217 and c-myc alone is not sufficient to cause immortalization, even if inhibition of telomerase by p53 is alleviated. We are currently examining other possible alterations that have occurred in these lines that may have contributed to their immortalization.

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Table 1: Methylation of hTERT promoter and hTERT/TRAP expression

Specimen ID/Cell type	Pass-age	hTERT methylation Dot-blot %	hTERT methylation MS-SSCA %	Genetic instability	hTERT mRNA	TRAP
Finite: Pre-stasis (M85-grown)						
184*	5	0	0	no	0	
184*-p16si	9	0	0	?	0	+/-
Finite: Post-selection						
184v-LXSN-Babe3	9	50	0	no	0	-
184v	12	1-5	0	agonescence	0	-
239 L E1-3	8	10	0	no	0	
239 L E1-3	13	5	0	agonescence	.00065	
161-LXSN	8	0	0	no	0	
161	9	0	0	no	0	-
48RO	6	0	0	no	0	-
Finite: Extended Life (BaP-exposed)						
184Aa	10	0	0	no	0	-
184Aa	16	0	0	agonescence	0	-
Finite: Post-selection transduced with ZNF217 +/or c-myc						
184v-ZNF-Babe2	9	60-70	0		0	-
184v-myc-LXSN1	9	60-70	0		0	-
184v-ZNF-myc1	9	60-70	0		0	-
184@K-ZNF	16	30-40	0		0	-
184@K-myc	15	80	50		.0023	+/-
239-ZNF	12	0	0		.00242	
161-ZNF	9	0	0		.00077	
Immortal Lines						
184[?@K or v]-HPV16E6	27	90	100	yes	.00043	
184@K-myc						
184@K-myc ***(finite)***	15	80	50		.0023	+/-
184@K-myc	29	90	50	yes	.0117	+++
184v-ZNF+myc						
184v-ZNF-myc1 ***(finite)***	9	60-70	0		0	-
184v-ZNF-myc1	21	0	0	no	.00026	
184v-ZNF-myc2	33	50	10	no	.0062	
184v-ZNF-myc3	20	20	0	no	2.51	+++
184-ZNF217 series						
184@K-ZNF ***(finite)***	16	30-40	0	no	0	-
184ZN4A	22	50-60	0	yes	.00083	-
184ZN4A	28	80	0	yes	.00539	++
184ZN4A	37	90	10	yes	.00463	nd
184ZN4A	48	100	100	yes	.00805	nd
184ZN5	31	80	50	yes	.0036	
184v-ZNF-Babe2 ***(finite)***	9	60-70	0	no	0	-

184v-ZNF (early jackpot)	22	0	0	no	54.25	+++
184Aa-derived lines (spontaneous)						
184A1	14	nd	0	no	.00081	-
184A1	56	nd	0	no	.0086	+++
184A1-myc	27	10-20	0		.0144	+++
184AA4	62	50	100	yes	.1077	++
184Aa-derived lines (spontaneous)						
184AA2 (p53-)	45	50	100	yes-high	.044	+++
184AA3 (p53-)	43	60-70	100	yes-high		+++
184Aa-derived (myc or ZNF217)						
184Aa-myc1	21	0	0		.000099	++
184Aa-myc2	21	90	100		.01	++
184AaZN1A	35	0	0	yes-low	.007	++
184AaZN1A	55	0	0	yes-low	.000144	++
184AaZN2A	39	0	0	yes-low	.018	++
184AaZN2A	53	0	0	yes-low	.345	++
184Be-derived line (spontaneous)						
184B5	43	90-100	100	yes-low	.00082	+++
184B5Y9H	100	10	0		0	-
184B5Y9H	112	10-20	50		.242	++

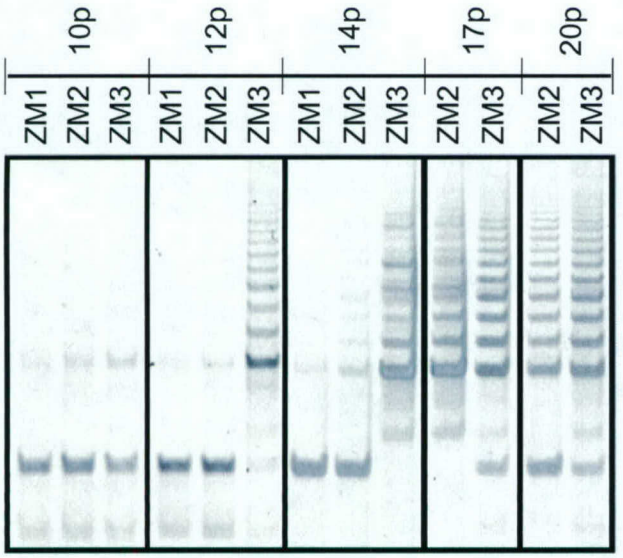

Fig. 1. Telomerase activity increases gradually at different rates in HMEC transduced with both ZNF217 and c-*myc*. Lysates of three independently transduced populations at indicated passages (p) were assayed for telomerase activity by the PCR-based TRAP assay. The presence of a ladder of PCR products of increasing size indicates positive telomerase activity.

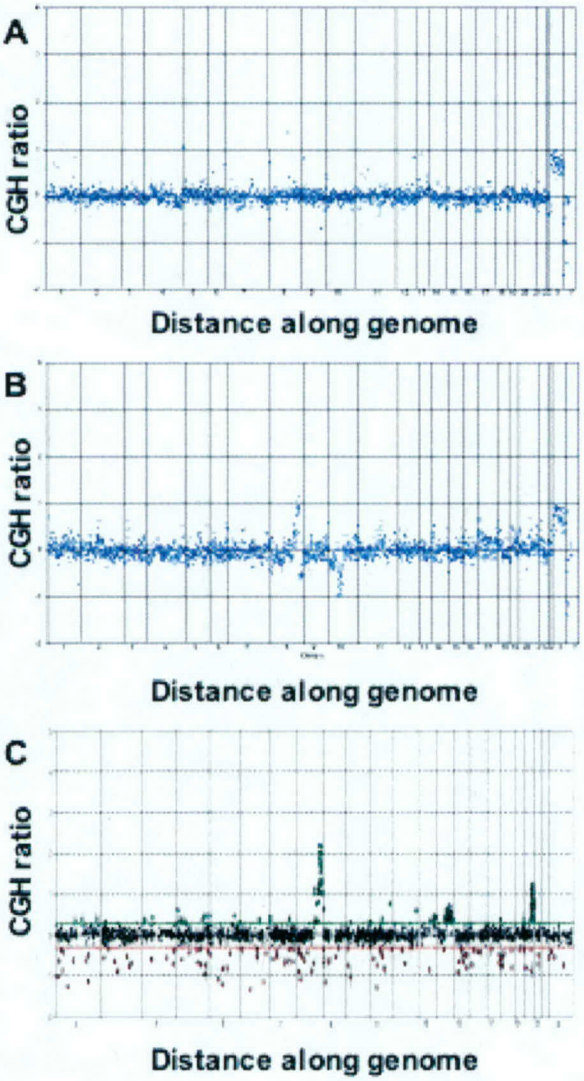

Fig. 2. Comparative genomic hybridization (CGH) results of immortalized HMEC lines (A) 184ZM3 passage 17, (B) 184ZM2 passage 17, and (C) 184-p16siRNA-LXSN passage 24. Note that CGH analysis showed no changes in gene copy number in 184ZM3, but amplification of the 8q locus where c-*myc* resides, as well as other alterations, in 184ZM2, and amplification of both 8q and 20q in 184-p16siRNA-LXSN.

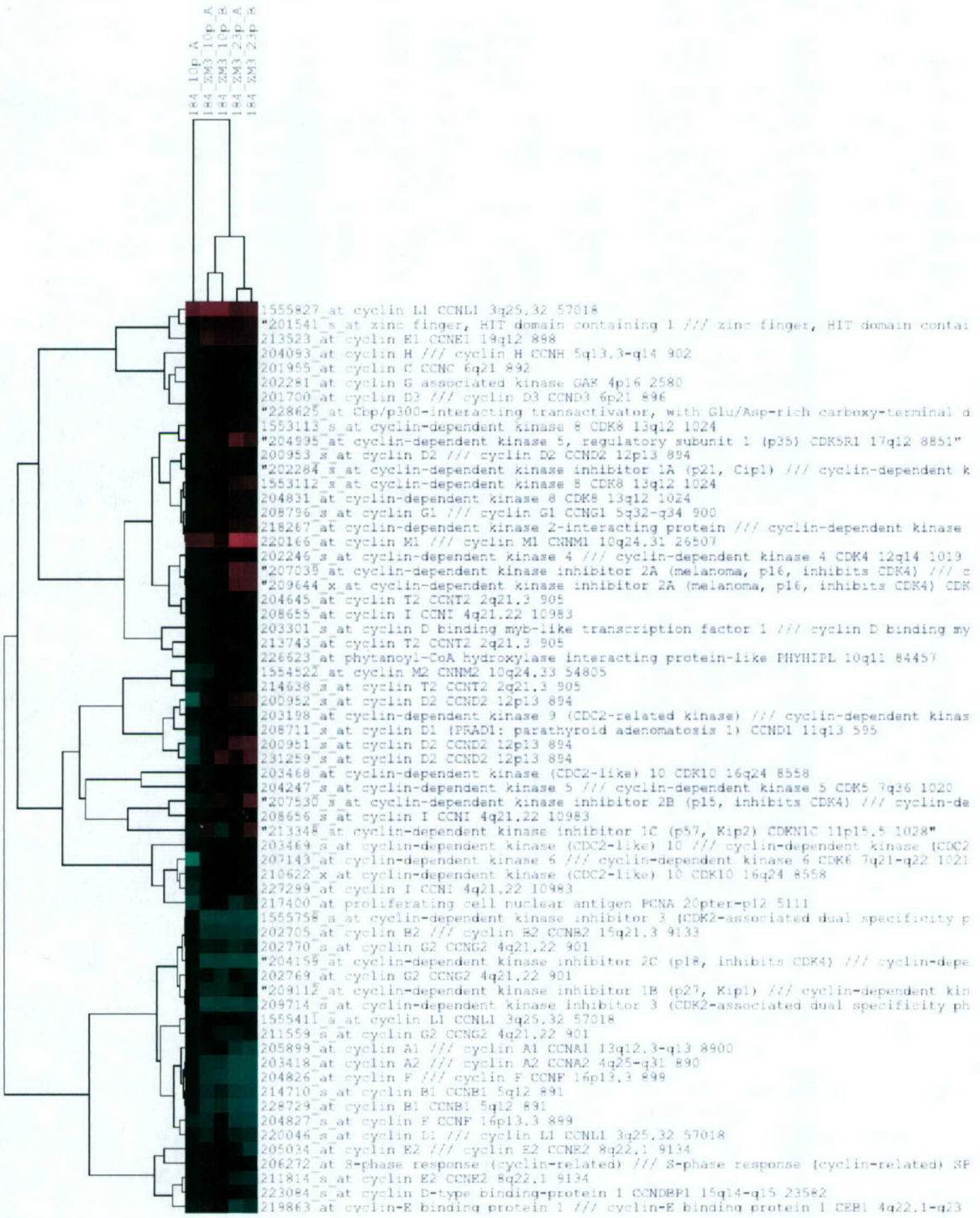

Fig. 3. Cluster analysis of selected cyclins/cdks/cdkis in parental finite lifespan 184, 184ZM3 at 10th passage prior to the de-repression of telomerase activity, and 184ZM3 at 23rd passage after the de-repression of telomerase activity.

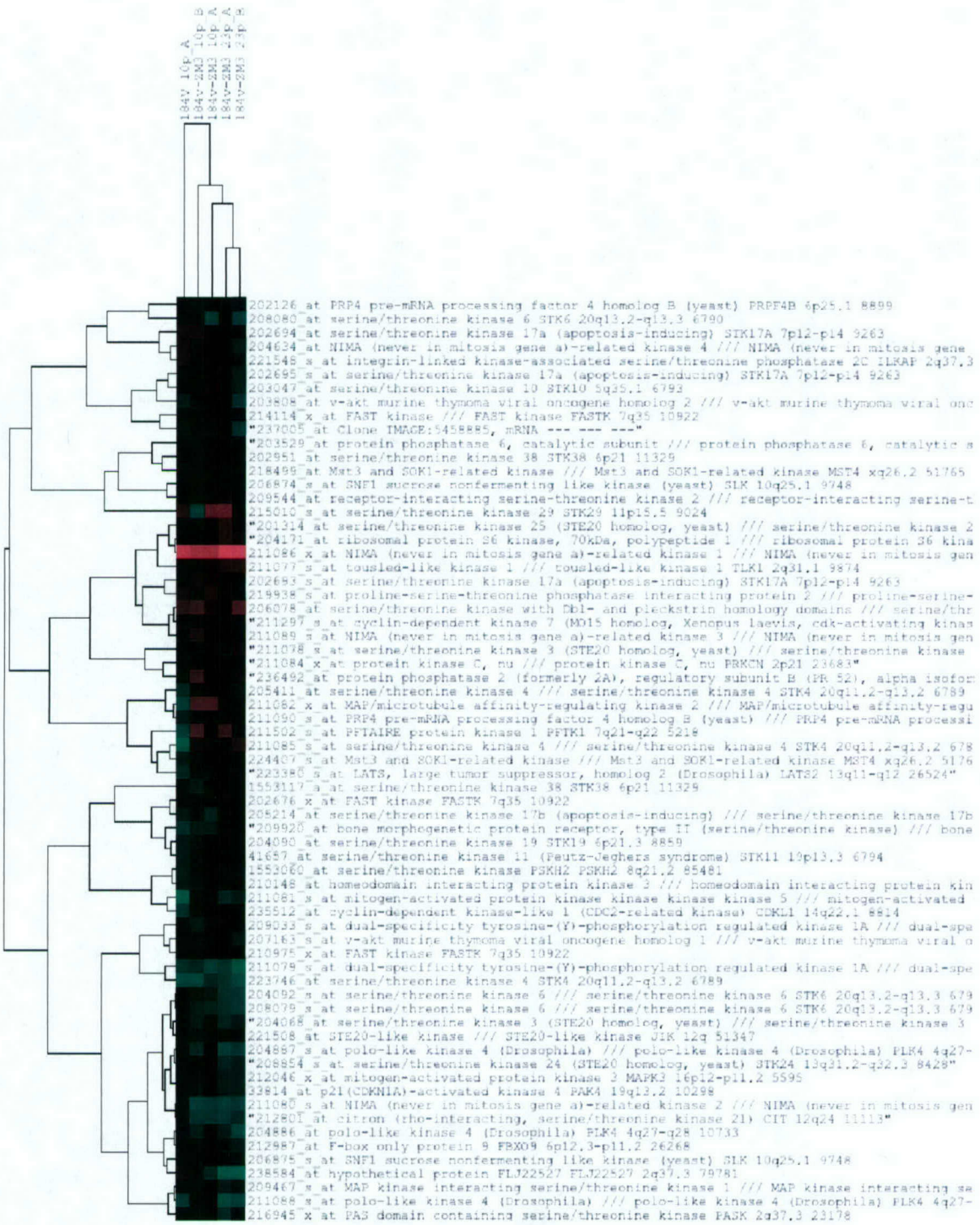

Fig. 4. Cluster analysis of selected serine/threonine kinases in parental finite lifespan 184, 184ZM3 at 10th passage prior to the de-repression of telomerase activity, and 184ZM3 at 23rd passage after the de-repression of telomerase activity.